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VARIABILITY OF STOMATA AND 45S AND 5S rDNAs LOCI CHARACTERISTICS IN TWO SPECIES OF *ANTHOXANTHUM* GENUS: *A. ARISTATUM* AND *A. ODORATUM* (POACEAE)

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Diploid *Anthoxanthum odoratum* and tetraploid *A. aristatum* were compared with respect to stomatal guard cell lengths, and stomatal density at adaxial and abaxial surfaces of the lamina. Further, the genome size of both species was determined by flow cytometry, and the number as well as the chromosomal distribution of 5S and 45S rDNAs were examined using FISH with ribosomal DNA (rDNA) probes. The average length of stomatal guard cells in *A. odoratum* was shown to be greater than that for *A. aristatum,* but the ranges overlapped. Moreover, reduction in stomatal frequency was found at higher ploidy levels.The genome size was 6.863 pg/2C DNA for *A. aristatum* and 13.252 pg/2C DNA for *A. odoratum*. *A. aristatum* has four sites of 5S rDNA in its root-tip meristematic cells, whereas *A. odoratum* has six. Both species have six sites of 45S rDNA. Chromosomal localization of the rDNA varied, which suggests that chromosome rearrangements took place during *Anthoxanthum* genome evolution.

Keywords: Ribosomal DNA – chromosome rearrangements – genome size – FISH

INTRODUCTION

Anthoxanthum L. is a large genus with cosmopolitan distribution that belongs to the Poaceae family. The genus contains about 15 species distributed in temperate and Arctic-Alpine Regions of Europe, Africa and Asia. In the flora of Poland, it is represented by only three of them, i.e., *A. alpinum, A. aristatum* and *A. odoratum* [46, 53]. However, they represent interesting objects of taxonomical, phytogeographical and ecological studies [7, 8, 29].

A. aristatum Boiss. (Annual Vernalgrass, 2n = 10) is an annual species, which originated from the western part of the Mediterranean region [36] and, since the 19th century, has been spreading fast through contiguous regions of Poland [28, 49]; in fact, it is regarded as invasive species. *A. odoratum* (Sweet Vernalgrass, 2n = 20) is a widespread Eurasian species [19, 41], which, in other regions of the world, especially in North America and Australia, is also considered an invasive species [5, 34].

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In Poland, *A. odoratum* occurs in a wide range of habitats, such as forests, dry grasslands, meadows, and ruderal habitats. It is classified as a native species that fully adapted to anthropomorphic conditions [20]. *A. odoratum* and *A. aristatum* frequently grow on neighbouring sites, especially in lowland habitats. Morphological studies of *A. odoratum* and *A. aristatum* populations revealed significant variations within these species [7, 8, 29], but occasionally morphological traits of both species overlap hampering their unambiguous identification. In order to find reliable traits for species discrimination, further anatomical investigations were planned, aiming for example to determine density and the size of stomata guard cells on the leaf blade, the features that can be used to determine ploidy level in grasses [23].

There is a dearth of complex karyological studies in the *Anthoxanthum* genus. The mitotic chromosome analyses done so far were limited to chromosome counts and morphology based on very simple, conventional methodology, such as staining with aceto-orcein. They revealed great variation in the number of satellite chromosomes, especially among *A. aristatum* and *A. odoratum* [24]. The comparative cytomolecular analysis of rDNA loci number and distribution in perennial *A. odoratum* (2n = 20) and annual *A. aristatum* (2n = 10) permits inferences on ploidy levels and provides insight into genome organisation of *Anthoxanthum* species.

Complex chromosomal rearrangements play a pivotal role in plant nuclear genome evolution at the level of chromosome. Genome structure and size can be affected by gene duplications and insertions. Tandem repeat duplications or segmental duplications are one of the possible outcomes of unequal cross-overs [54]. Highly repetitive DNA sequences often form large loci along chromosomes, and their detection using fluorescence *in situ* hybridisation (FISH), the molecular cytogenetic method that enables to physically map DNA sequences of interest and directly visualise them under the microscope, is relatively simple [11, 40]. Genes encoding for 5S ribosomal rRNA as well as 18S, 5.8S, 25S rRNA, commonly known as 5S rDNA and 45S rDNA, are most frequently studied by FISH (http://www.plantrdnadatabase.com/). They occur at one or more chromosomal loci and their coding regions are highly conserved among various organisms, while the non-coding ones are much more variable and often exhibit variation sufficient to allow examination of phylogenetic interrelationships [51]. In plant genomes, rRNA genes can rapidly change in copy number and chromosomal location. Therefore they provide valuable cytogenetic insight into genome organisation, expressed *inter alia* by the loss and/or gain of rDNA loci and probably also by changes in rDNA unit copy number among related species [18, 42]. They have been successfully used as robust chromosome-specific landmarks FISH probes targets in various plant genera, including *Avena* [31], *Brachypodium* [52], *Hordeum* [48], *Rhoeo* [12] and *Oryza* [4], in many cases providing useful, chromosome-specific landmarks.

Considering limited information that exists on the genome structure in diploid and tetraploid species of *Anthoxanthum,* FISH with ribosomal rDNA-based probes was used to: (1) determine the number and chromosomal distribution of both 5S and 45S rDNAs; (2) track possible mechanisms of *Anthoxanthum* genome evolution at the chromosome level; (3) establish chromosomal markers for further detailed karyological analyses.

MATERIALS AND METHODS

Plant material

A. odoratum and *A. aristaum* were collected in nature in various locations. Details on the origins of this plant material are given in Table 1.

Table 1
Origins of *A. odoratum* and *A. aristatum* samples

Species	Samples	Location
A. odoratum	1, 2, 3	Chlebowo (Wielkopolska region, western Poland)
A. odoratum	4, 5	Morasko (Wielkopolska region, western Poland)
A. odoratum	6, 7	Babia Góra Massif, Beskidy Mountains (Małopolska region, southern Poland)
A. odoratum	8, 9, 10	Sandr Nowotomyski (Wielkopolska region, western Poland)
A. odoratum	11, 12, 13, 14	Lublin Upland (Lublin region, eastern Poland)
A. aristatum	15, 16	Chlebowo (Wielkopolska region, western Poland)
A. aristatum	17, 18, 19	Morasko (Wielkopolska region, western Poland)

Measurements of stomata

Plant material was fixed in 70% ethanol. For stomata size and density measurements, the third leaf below the panicle was sampled from each plant. Epidermis was removed from the adaxial and abaxial part of the lamina. Sections were mounted in a chloral hydrate solution and examined with bright field microscopy. Stomatal guard cells length on both surfaces were measured with Lucia G software. Stomatal density (number of stomata per mm^2) was also evaluated on both surfaces of the lamina. In each sample, 3 plants were analysed; 30 guard cells on each surface of the lamina were measured. The results were statistically processed using Statistica software. In order to determine statistical significance of average values, the factor variance ANOVA F-statistics was used [33, 38].

Mitotic chromosome preparation

Chromosome slides were prepared from the root meristem tissue according to the protocol for mitotic chromosome squashes provided by Jenkins and Hasterok [22] with minor adjustments specific to the *Anthoxanthum* material. Briefly, the seeds were germinated on moistened filter paper in Petri dishes at room temperature. Seedlings

with approximately 2.0 cm root length were treated in ice-cold tap water for 24 h. Excised roots were drained and immediately fixed in freshly made methanol:glacial acetic acid (3:1) and stored at –20 °C until use. Roots were digested in 20% (v/v) pectinase (Sigma, St. Louis, MO, USA), 1% (w/v) cellulase (Sigma, St. Louis, MO, USA) and 1% (w/v) cellulase "Onozuka R-10" (Serva, Heidelberg, Germany) mixture for 2–3 h at 37 °C. Chromosome preparations were made from dissected meristematic tissue on alcohol-cleaned slides in one drop of 45% acetic acid, frozen on dry-ice and then air dried. The slides were quality-controlled under a phase-contrast microscope and used for FISH.

DNA probes and FISH

To obtain the 5S rDNA probe, the wheat clone pTa794 [10] was labelled with tetramethyl-rhodamine-5-dUTP (Roche Diagnostics, Basel, Switzerland) by PCR according to the protocol by Hasterok et al. [15]. The 25S rDNA probe was generated using 2.3-kb *Cla*I subclone of the 25S rDNA coding region of *Arabidopsis thaliana* [50] and labelled with digoxigenin-11-dUTP (Roche Diagnostics) by nick translation as described by Jenkins and Hasterok [22]; FISH was also performed using this same protocol. Briefly, the slides were pre-treated with RNase (100 µg/ml) in 2× SSC in a humid chamber at 37 °C for 1 h. The hybridisation mixture (50% deionised formamide, 10% dextran sulphate, 2× SSC, 0.5% SDS, sonicated salmon sperm DNA in 25–100× excess of the probe, 75-200 ng probe/slide) was denatured at 90 °C for 9 min, then applied to the chromosome preparation and denatured together at 75 °C for 4.5 min using an Omnislide *in situ* hybridization system (Hybaid, Basingstoke, UK). Hybridisation was carried out in a humid chamber at 37 °C for 20–24 h. Immunodetection of the digoxigenated probe was carried out with FITC-conjugated anti-digoxigenin primary antibodies (Roche Diagnostics). Chromosomes were counterstained with 2 µg/ml DAPI in Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). Slides were examined with an Olympus Provis AX epifluorescence microscope. The images were captured using a CCD monochromatic camera, tinted in Wasabi (Hamamatsu Photonics) and superimposed using Micrografx (Corel) Picture Publisher 10 software.

Genome size estimation

Flow cytometry was carried out as described in Hajdera et al. [13] using a DAKO Galaxy flow cytometer equipped with an air-cooled argon ion laser. The suspensions of nuclei were prepared from young leaves and then stained with PI (propidium iodide). For *Anthoxanthum* genome size estimation, *Pisum sativum* cv. Citrad genome (9.09 pg/2C DNA; D. Siwinska, pers. comm.) was used as the standard. The data were processed using FloMax (Partec GmbH).

RESULTS AND DISCUSSION

Stomatal guard cell characteristics

The lengths and density of stomatal guard cells, on both adaxial and abaxial surface of lamina – (traits 1, 2, respectively) showed significant variability in the two species (Fig. 1). Values F were statistically important at the level $p<0.001$ for these traits. The longest stomata were noted in the tetraploid species *A. odoratum* and ranged from 29.3 µm to 54.9 µm, the shortest stomata occurred in the diploid *A. aristatum* and ranged from 32.7 µm to 37.3 µm. Stomatal length was significantly correlated

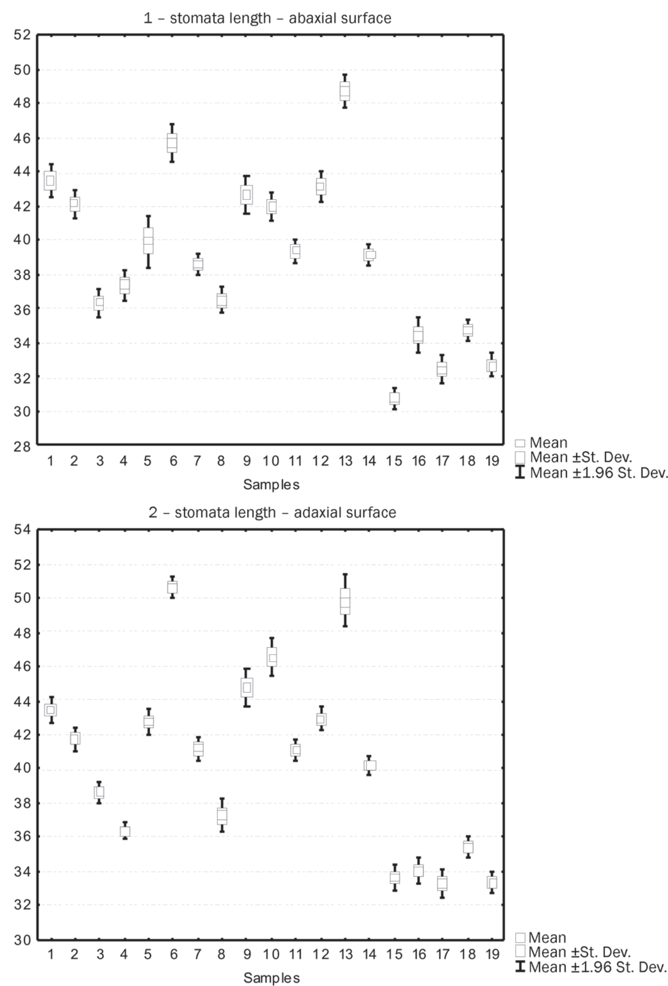

Fig. 1. Box plots for comparing samples of *A. odoratum* (1–14) and *A. aristatum* (15–19) according to stomata length on the abaxial and adaxial surface of lamina (respectively traits 1 and 2)

with the ploidy level. The results agree with those previously reported for other grass species, including *Triticum* ssp. [25] and *Phleum* sect. *Phleum* [23]. However, our results indicate that stomatal length cannot be considered as a perfect marker for ploidy level determination within the *Anthoxanthum* genus, because the length of stomata in two examined species *A. odoratum* and *A. aristatum* overlapped. In *A. odoratum,* the density of stomata ranges from 87 to 92 per 1 mm^2 on the adaxial and from 48 to 59 on the abaxial surface of the lamina, while in *A. aristatum* from 101 to 125 and 63 to 79, respectively. The species differ in their stomatal density and, furthermore, correlation was noted between stomatal density and length. The reduction in stomatal frequency at higher ploidy levels resulted mainly from the presence of larger epidermal cells. Similar observations have been reported [2, 37]. Stomata are associated with many physiological functions, so reduction or increase in the number of stomata can also be caused by environmental factors [47].

Ribosomal DNA loci pattern and nuclear genome size

Significant variation in numbers and localisation patterns of rDNA loci even among closely related species has been noted for many different genera, including *Brassica* [18], *Phaseolus* [39] and *Nicotiana* [26]. The variation in the loci number can occur for both classes of rRNA genes, as was demonstrated within the *Paphiopedilum* subgenus, or for 45S rDNA but not for 5S rDNA in *Parvisepalum* subgenus [27]. Conversely, in *Medicago* species, 45S rDNA loci number is constant, while the number of their 5S rDNA counterparts varies from 1 to 4 [45]. Genome organisation and chromosomal structure in the *Anthoxanthum* genus is poorly investigated and no cytomolecular study has been done so far. Application of FISH using ribosomal DNA probes allowed determination of the number and distribution of 45S rDNA and 5S rDNA loci in *A. aristatum* and in *A. odoratum* (Table 2). Our study revealed that *A. aristatum* and *A. odoratum* have, respectively, four and six sites of 5S rDNA. Six sites of 45S rDNA were found in each species.

Chromosomal localization of both rDNA sites varied between *Anthoxanthum* species. In *A. aristatum,* four sites of 45 rDNA were located in the subterminal regions of two chromosome pairs (Fig. 2A). The third pair of chromosomes possessed two 45S rDNA sites in distal chromosomal positions located between four 5S rDNA sites (Fig. 2A, marked by arrows). Similarly, *A. odoratum* had four sites of 45 rDNA, two

Table 2
The number of rDNA sites and nuclear genome size of *Anthoxanthum* species

Species	Chromosomes number (2n)	Number of rDNA sites			Genome size (pg/2C DNA)	Standard deviation
		5S	45S	(5S + 45S)		
A. aristatum	10	–	4	(4+2)	6.863	0.045
A. odoratum	20	4	4	(2+2)	13.252	0.061

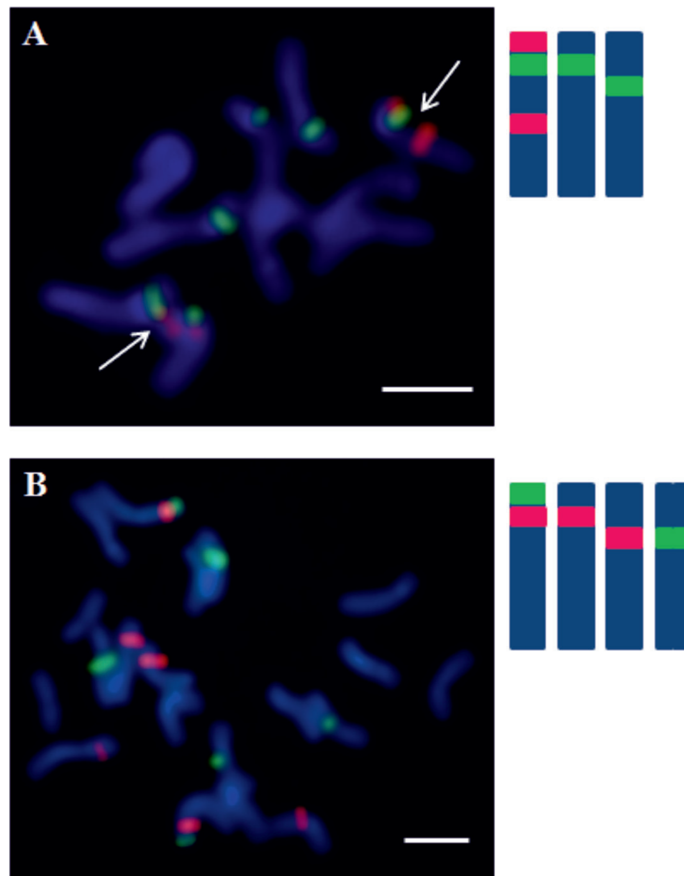

Fig. 2. Chromosomal location 5S rDNA (red) and 25S rDNA (green) in *A. aristatum* (A) and *A. odoratum* (B). Chromosomes are counterstained with DAPI (blue). Arrows indicate the chromosome pair carrying 1 locus of 45S rDNA and 2 loci of 5S rDNA. Bars: 5 μm. The ideograms of chromosomes with 5S and 45S rDNA are shown on the right

of which also mapped in subterminal regions of two chromosome pairs. One pair of chromosomes bears the remaining two sites of 45S rDNA as well as two of the six sites 5S rDNA in distal regions. The remaining four sites of 5S rDNA are distributed in proximal regions of two other pairs of chromosomes (Fig. 2B).

In *Anthoxanthum,* analysis of rDNA loci number and distribution provides valuable insight into genome evolution at the chromosomal level. Comparison of localisation of 45S rDNA sites, closely linked with either two or four 5S rDNA sites on one chromosome pair indicates that chromosome rearrangements took place during *Anthoxanthum* genome evolution. Different mechanisms, such as transposon-mediated transpositional events [1, 43], various chromosome rearrangements caused by homologous or non-homologous unequal crossing-over, and gene conversion [30,

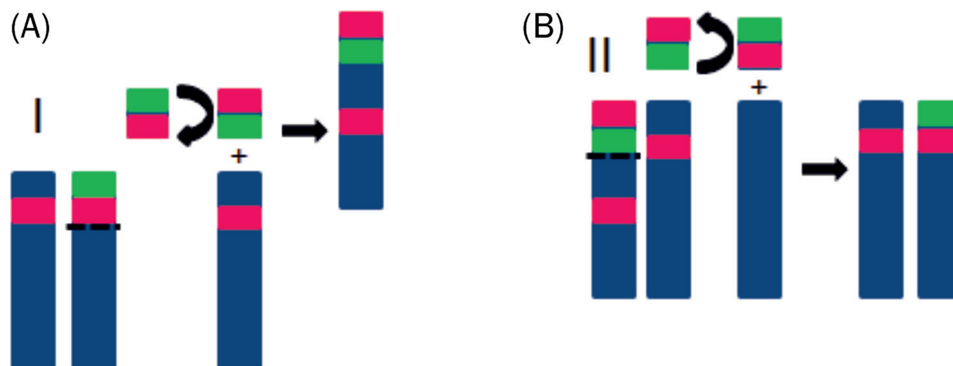

Fig. 3. Possible mechanism of chromosomal rearrangements that lead to one chromosome with 2 sites of 5S (red) and one site of 45S rDNA (green) (A), or to two chromosomes with one 5S site and another with both rDNA types (B)

44] could act alone or in combination. While such events do not necessarily impart changes in overall chromosome morphology, they can contribute to the variation of numbers, sizes and localisation of rDNA sites. One of the possibilities is that translocation of 45S and 5S rDNA sites localised on the same *A. odoratum* chromosome pair took place and inversion of the translocated region then occurred, thereby establishing one pair of *A. aristatum* chromosomes (Fig. 3A). However, it cannot be ruled out that translocation and inversion of 45S and 5S rDNA sites in one chromosome of *A. aristatum* led, in *A. odoratum,* to one chromosome pair with only one site of 5S rDNA and one chromosome pair with both 5S and 45S rDNA (Fig. 3B). Additionally, the difference in number of 5S rDNA sites can also indicate chromosome rearrangements within these species. The loss of 5S rDNA sites could reflect complete elimination of entire sites, or perhaps only a significant reduction of copies of 5S rDNA repeats at the respective site(s), resulting in a diminished signal or leaving too few repeats in a locus to be detected by FISH. Such outcomes could be attributed to spontaneous deletion of an rDNA-bearing fragment from one chromosome arm, or to unequal crossing over which could lead to a loss of repeats from different sites.

Ribosomal DNA sequences are commonly used as effective chromosome landmarks, for example to identify alien chromosomes in addition lines [17] thereby revealing phylogeny among closely related species, including interspecific hybrids [14]. They can also be used to detect polyploidisation events and to track various chromosome rearrangements that occur naturally during plant nuclear genome evolution [16, 18] as well as under experimental induction [35].

Use of two rDNA-based probes distinguished three pairs of chromosomes in *A. aristatum* and five in *A. odoratum*. Ribosomal DNA sequences were successfully used for identification of all chromosomes in *A. thaliana* [9] and of some chromosomes in many other species, e.g. within the genus *Fragaria* [32], *Pinus* [3], and

Citrus [6]. Moreover, close linkage of 45S and 5S rDNA on the same chromosome pair can be used as an unambiguous marker for species identification. To our knowledge, the present results provide the first specific cytomolecular markers for *Anthoxanthum* chromosomes and can be helpful for further karyological study of those species.

In the search to reveal the evolutionary trends in the variation of nuclear DNA content and the biological significance thereof, efforts have been made to estimate DNA amounts in *Anthoxanthum* species. The genome size of *A. aristatum* as determined by flow cytometry is 6.863 pg/2C DNA, whereas for *A. odoratum* it is 13.252 pg/2C DNA. A previous study using flow cytometry established the DNA content of *A. odoratum* to be 11.2 pg/2C DNA (Kew Garden C-value database, http://data.kew.org/cvalues/CvalServlet?querytype=1 (Table 2). This difference may be attributed to different methodologies and to a different DNA standard. Here, we report for the first time the genome size of *A. aristatum* (6.863 pg/2C DNA). Chromosome number can be positively correlated with genome size [21, 52]. Indeed, the *A. aristatum* genome (2n = 10) is about two-fold smaller than that of *A. odoratum* (2n = 20). The genome size and number of rDNA loci suggests that complex rearrangements which involved either insertion or deletion of DNA segments have reshaped the *Anthoxanthum* genomes.

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